AD_____

Award Number: DAMD17-02-1-0487

TITLE: Investigation of the Role of Sialomucin Complex (SMC)/Muc4, a Unique Intramembranous HER-2/ErbB-2 Ligand, as a Suppressor of Apoptosis

PRINCIPAL INVESTIGATOR: George Theodore

CONTRACTING ORGANIZATION: University of Miami School of Medicine
Miami, Florida 33136

REPORT DATE: April 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2003	Annual Summary (1 Apr 2003 - 31 Mar 2004)

4. TITLE AND SUBTITLE
Investigation of the Role of Sialomucin Complex (SMC)/Muc4, a Unique Intramembranous HER-2/ErbB-2 Ligand, as a Suppressor of Apoptosis

5. FUNDING NUMBERS
DAMD17-02-1-0487

6. AUTHOR(S)
George Theodore

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Miami School of Medicine
Miami, Florida 33136

E-Mail: gtheodor@newssun.med.miami.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
No Abstract Provided.

14. SUBJECT TERMS
No Subject Terms Provided.

15. NUMBER OF PAGES
7

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....

SF 298.....1

Table of Contents.....2

Training.....3

Key Research Accomplishments.....3

Annual Summary Report for DAMD17-02-1-0487
George Theodore

TRAINING
Wrote and defended thesis proposal successfully
Took and passed the Seminar and Journal Club course in the Department of Cell Biology and Anatomy

RESEARCH ACCOMPLISHMENTS
Introduction
Muc4/sialomucin complex (SMC) is a high M_r heterodimeric glycoprotein complex which was originally observed at the cell surfaces of 13762 rat mammary adenocarcinoma cells and has been more recently found in many accessible and vulnerable epithelia. It is composed of a mucin subunit ASGP-1 and a transmembrane subunit ASGP-2. The latter has two EGF-like domains and can form intramembrane ligand-receptor-type complexes with the receptor tyrosine kinase ErbB2. An important aspect of SMC/Muc4 is its ability to repress apoptosis when transfected into tumor cells. Our hypothesis is that SMC/Muc4 is multifunctional. It acts as an epithelial protective agent by forming a steric barrier at epithelial apical surfaces and by contributing to signaling through ErbB2 involved in epithelial differentiation and repression of apoptosis. Both of these functions may contribute to tumor progression when Muc4/SMC is inappropriately overexpressed.

HER-2 (ErbB-2, neu) is a member of the family of epithelial growth factors (EGF), and appears to overexpressed in 25-30 % of human breast carcinomas. Its presence is associated with a poor prognosis. No soluble ligand for HER-2 has been discovered so far, but our research group has shown that the ASGP-2 subunit of Muc4 binds to HER-2 (ErbB-2, neu), and affects its phosphorylation. Muc4 has also shown to suppress apoptosis in A375 human melanoma cells and to upregulate a differentiation-associated protein $p27^{kip1}$. High levels of expression of $p27^{kip}$ have been seen in some highly proliferative human breast cancer cells. Thus the study of HER-2-Muc4 complex in regards to its suppression of apoptosis will provide us with a more complete understanding of the mechanisms by which these molecules participate in breast cancer; this in turn we hope, will ultimately contribute to the refinement of breast cancer therapies.

Task 1. To detect the interaction of HER-2 (ErbB-2) and Muc4 with endogenous cellular proteins in cultured cells
The most important recent discovery from our laboratory is the finding that Muc4 transfected into polarized epithelial cells can induce the relocalization of ErbB2 from the lateral surface to the apical surface. This relocalization segregates the ErbB2 from its co-receptor ErbB3. Heterodimerization of the ErbB2 and ErbB3 is an important mechanism in the activation of the receptors in ErbB2/HER2/Neu-driven breast cancers. The segregation imposed by Muc4 is lost in neoplastic cells, which have lost their polarization as a result of oncogenic transformation.

To determine whether this segregation mechanism applies to normal epithelia, we have examined the localization of ErbB2 in rat mammary gland and human airway epithelia. Since our previous studies have shown that different anti-ErbB2 antibodies recognize different forms of the receptor with different localizations, we have used multiple antibodies in these studies. The most representative results are shown.

To compare cellular localizations of Muc4/SMC and ErbB2 in lactating mammary gland and to compare ErbB2 staining pattern with different anti-ErbB2 antibodies, whole mammary tissue isolated from lactating rats was analyzed by immunohistochemical staining with antibodies directed against Muc4/SMC (ASGP-2) and ErbB2. As expected, Muc4/SMC was stained on the apical surfaces of both ductal and alveolar epithelial cells. When stained with Neomarkers anti-ErbB2 antibody 1, a polyclonal antibody directed against a peptide in the C-terminal region of ErbB2, ErbB2 was detected at the apical surfaces of the ductal and alveolar epithelial cells, though the latter was poorly stained (Fig. 1). However, when stained with Dako anti-ErbB2 (Herceptest), ErbB2 staining was localized largely on the lateral surfaces of the alveolar and ductal epithelial cells, though the latter were poorly stained. No staining was detected with any of the antibodies in the myoepithelial or stromal cells.

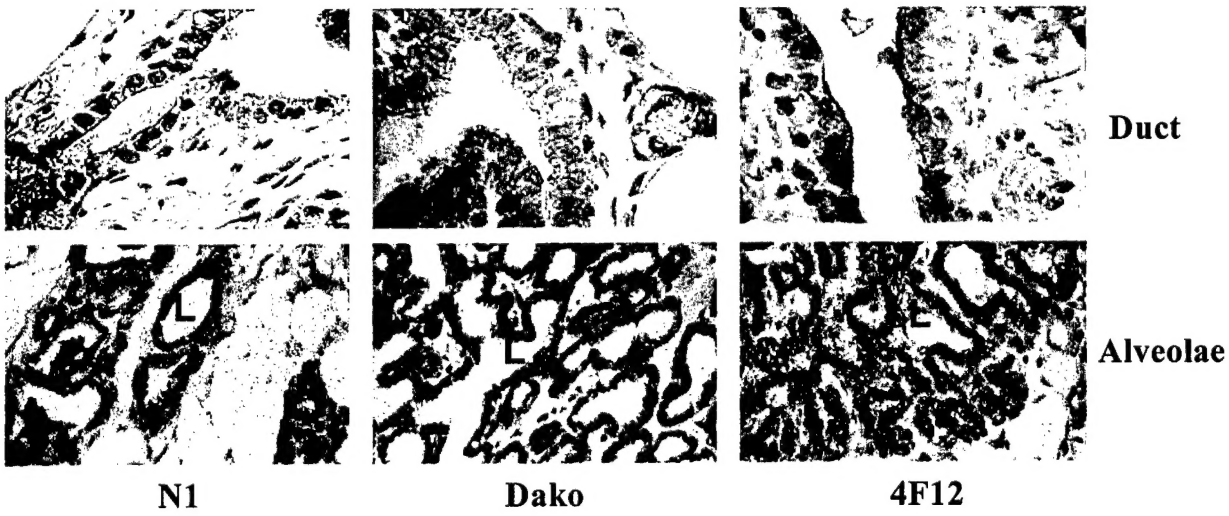

Figure 1. *Localization of Muc4/SMC and ErbB2 in lactating mammary gland. Sections (5 μm) of lactating rat mammary gland were stained with Dako anti-ErbB2, NeoMarkers anti-c-neu antibody 1 (N1), or anti-ASGP-2 mAb 4F12 as indicated in the figure. The specifity of these antibodies and the staining controls were demonstrated in a previous study. L designates lumens of alveoli.*

A similar result was obtained with human airway tissue, which is a pseudo-stratified epithelium. In this case staining with antibodies to Muc4 and ErbB2 were observed predominantly at the apical surfaces of the epithelia. Little or no staining was observed with the Dako antibody, which usually stains basolateral forms of the ErbB2.

These studies present a new aspect of the regulation of ErbB2, regulation by localization in polarized epithelial cells. Muc4 localizes ErbB2 to the apical surfaces of epithelia and limits its phosphorylation. The segregation from ErbB3 limits the phosphorylation of this receptor and restricts activation of the PI3K-Akt pathway, which is stimulated by ErbB3 phosphorylation. Akt activation has been shown to be an important event in breast cancer oncogenesis. Our hypothesis is that ErbB2 acts as a switch between epithelial differentiation and proliferation and that Muc4 regulates that switch by its effects on ErbB2 localization and signaling.

Task 2. To investigate the hypothetical link between HER-2 (ErbB-2)-Muc4 complex formation and suppression of apoptosis by Muc4

Our attempts to determine the link between ErbB2 and apoptosis have not been successful, as the methods that we have used for blocking ErbB2 expression have direct effects on apoptosis. We have put this aim aside at present to work on other aspects of the project. We plan later to investigate methods for blocking ErbB2 activity without blocking its expression.

Task 3. To determine which pathway of caspase activation is inhibited by Muc4-HER-2 (ErbB-2) complex

The repression of apoptosis by Muc4 raises the question of the mechanism by which Muc4 acts. To begin to address this question, we analyzed caspases associated with the two primary pathways linked to apoptosis. Caspase 9 and caspase 8 are key elements of the intrinsic and extrinsic pathways, respectively. Caspase 9 activation was strongly inhibited by expression of Muc4 when cells were treated with the apoptotic agent actinomycin D. In contrast, Muc4 did not repress activation of caspase 8 when the extrinsic pathway was induced. Caspase 9 can be activated by either association or by cleavage. To determine whether Muc4 is able to block caspase 9 cleavage, we used antibodies which recognize both the intact and cleaved form of the protease. Cells were grown under conditions to limit attachment and induce anoikis (apoptosis due to loss of adhesion). Parallel cultures were treated with or without tetracycline to induce expression of Muc4. Caspase 9 cleavage was observed in the cultures undergoing anoikis, and Muc4 expression repressed both the cleavage and anoikis.

These results clearly show that Muc4 blocks the intrinsic (mitochondrial) pathway. We are now seeking to identify elements further upstream in this pathway which are susceptible to Muc4 expression. Recent studies have concentrated on members of the Bcl family of apoptosis effectors. A preliminary finding suggests that the pro-apoptotic family member Bim is upregulated in cells undergoing anoikis, but down-regulated when those cells are induced to express Muc4. Additional studies are underway to verify these results and examine other Bcl family members.

Task 4. To investigate the contribution of $p27^{kip}$ in suppression of apoptosis by Muc4-HER-2 (ErbB-2) complex

One mechanism for Muc4 repression of apoptosis is via $p27^{kip}$, which has been shown to be involved in inhibition of apoptosis in some systems. However, blockage of $p27^{kip}$ expression using an antisense approach did not inhibit the ability of Muc4 to repress apoptosis. Thus, $p27^{kip}$

does not appear to be involved in the Muc4 repression of apoptosis. This result completes this task with a negative conclusion and ends research on this aim.

Task 5. Investigate the regulation of $p27^{kip}$ expression by Muc4
Work on this task has not yet begun.

Summary. The work during this period provides further evidence for a new mechanism for the regulation of ErbB2, a key factor in about 25% of breast cancers. This mechanism may play a key role in breast cancer progression in these cancers.